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In-House Fabrication of Multi-Sized Polystyrene Microcarriers for Neural Stem Cell Expansion

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IN-HOUSE FABRICATION OF MULTI-SIZED POLYSTYRENE MICROCARRIERS FOR NEURAL STEM CELL EXPANSION

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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ABSTRACT

IN-HOUSE FABRICATION OF MULTI-SIZED POLYSTYRENE MICROCARRIERS FOR NEURAL STEM CELL EXPANSION

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The clinical demand for neural stem cells drives the need for a reproducible method used to generate a large quantity of well-characterized cells to support regenerative therapies. Microcarriers are currently being used as a scaffold for stem cell culture to aid in this expansion process because they provide a larger surface area while also requiring fewer cell passages compared to monolayer culture. The main objective of this project was to investigate how human neural stem cell attachment, proliferation, and multipotency would respond to three-dimensional (3D) culture using peptide-coated microcarriers of varying diameters. In this study, multi-sized polystyrene microcarriers were fabricated

using a vibration generator and solvent evaporation techniques in order to optimize the size distribution and homogeneity of the scaffold. Overall, the results indicated that when supplemented with a beneficial surface coating, the constructed microcarriers were successful at promoting stem cell attachment, growth, and viability without interference from surface curvature. This research will provide insight for further studies involving the feasibility of 3D culture on neuronal differentiation, the scale-up advantage of microcarriers, and clinical therapies.

1. INTRODUCTION

Regenerative medicine is an interdisciplinary field that combines stem cell therapies and tissue engineering in order to treat disorders through safely restoring and repairing damaged organs[1]. Stem cell therapies are anticipated to treat a number of conditions including cancer, Parkinson's disease, cardiac failure, neurological disorders, and Type 1 diabetes[2]. Even with the continuing advancements with stem cell research, there is still a set of drawbacks that can hinder the routine use of cell treatments. One major limitation for stem cell based therapies is the shear number of cells needed for effective transplantation doses. When considering an effective treatment for Parkinson's disease, an individual transplantation should allow for the survival of 10^5 grafted dopaminergic neurons[3]. In a study involving autologous peripheral-blood stem cell transplantation, an effective dose for neutrophil recovery was between 5×10^6 and 8×10^6 cells per kg of the patient[4]. Finally, for the clinical use of amniotic fluid stem cells (AFSCs), around 10^9 cells are needed for a patient weighing 70 kg[5]. With two dimensional (2D) cell culture allowing for only 10^5 cells/mL, about 10,000 mL of traditional cell suspension is needed for one transplantation dose[5]. Therefore, designing a scalable process for cell expansion independent of traditional cell culture procedures would be extremely beneficial when producing clinically relevant numbers for cell transplantation.

Typically in research laboratories cell lines are grown on flat surfaces, including flasks and plates. Although 2D cell culture is widely accepted, it does not fully represent the natural environment of cells within biological structures such as tissues and organs. Cells that make up these three-dimensional (3D) structures form a multifaceted biochemical and mechanical network through cell-to-cell interactions as well as connections to the extracellular matrix (ECM)[6,7]. Integrin receptors located on the cell's surface allow the cells to link to the ECM, providing not only mechanical support but also an avenue for communication for important physiological cues involving cell growth, survival, organization, migration, and differentiation[6,8]. The geometry of flat substrates reduces the possibility of cells forming these multidimensional networks, so there needs to be a bridge between the traditional *in vitro* cell culture experiments and *in vivo* studies.

Growing cells three dimensionally *in vitro* has its advantages for multiple applications including cell expansion, drug discovery, and cancer research because it allows cells to grow in an environment that is closer to *in vivo* testing[9]. Using 3D cell culture systems gives a model prior to *in vivo* models to overcome the limitations of 2D cell culture, which can be extremely beneficial since animal studies are typically costly as well as practically and ethically difficult [10,11]. In addition to the geometrical differences between 2D and 3D cell culture systems, when considering cell expansion, monolayer cell culture in petri dishes or flasks is limited by surface area, labor intensive, difficult to scale-up, and prone to contamination[5]. 2D static culture systems are suitable for small-scale projects but when considering the enormous quantity of stem cells needed for effective therapies there needs to be a more efficient way to increase scalability.

There are various methods of 3D cell culture systems that have been used in literature, ranging from cell spheroid formation to scaffold-based approaches. These methods are outlined in Table 1.2, including their advantages and disadvantages. Originally spontaneous spheroid formation, such as forced-floating, hanging drop, and spinner flask methods, were used to monitor 3D cell growth and now various scaffold options are gaining popularity[10]. Cell spheroids allow for greater cell scalability when compared with monolayer culture and can mimic the stem cells' native microenvironment by cell-cell and cell-ECM connections[3]. However, because of the cell-to-cell contacts within the spheroid, spontaneous differentiation can occur and there can be cell death in the center of the sphere when the diameter becomes too large[3]. This will require dissociation and re-aggregation steps throughout the cell expansion process to prevent agglomeration, adding additional labor and complexity of this 3D cell culture system[3].

Scaffolds are mainly used for clinical applications and 3D laboratory modeling, both of which have their own set of requirements. Clinical applications of scaffolds consist of creating practical implants that not only maintain tissue function but can also be metabolized into the body without creating an adverse reaction while *in vitro* models are used to improve the knowledge of cell biology and tissue physiology by mimicking the 3D nature of the body[12]. Important parameters are taken into consideration when creating scaffolds including porosity, elasticity, biocompatibility, mechanical stability, support of cell attachment, and diffusion of nutrients and waste in and out of the scaffold[13,14]. These aforementioned properties can be adjusted depending on the technique used during the fabrication process. It is also common to include ECM

proteins, such as laminin or collagen, to 3D cell culture systems in order to promote the cell-to-ECM connections characteristic of naturally occurring three-dimensional structures. When considering 3D scaffolds for cellular expansion, fibers, hydrogels, and microcarriers were further explored.

Fibrous scaffolds are manufactured using electrospinning, self-assembly, or phase separation methods with electrospun scaffolds being the most widely studied technique for various applications[15]. Fibers created by electrospinning are done so by forcing a polymer solution through a syringe that is connected to a voltage source, charging the solution[16]. When the electrostatic force overcomes the surface tension of the liquid, a polymer jet stream is produced from the top of the syringe that then travels and dries in flight to the ground collector[16]. Electrospinning has the capacity of adjusting the size of the nanofibers within the scaffold, ranging from 100-500 nm, which is a major advantage because the fabricated fibers closely mimic the size of fibrous proteins found in the ECM, giving cells an environment that is similar to naturally occurring structures[16,17]. The increase in surface area-to-volume ratio as well as the microporous nature of fibrous scaffolds allows for an increase in cell attachment, migration, proliferation, and differentiation, which are very important when considering stem cell expansion for cell transplantation applications[15,17]. However, fibrous scaffolds are limited to their thickness and small pore size, which can prevent cell migration into the scaffold[17]. Alterations to fabrication techniques have been used to overcome these limitations, such as using ultrasonification in order to increase pore size to 10 microns or larger to allow for a greater cell infiltration[17]. Use of multilayer electrospinning has also help improve the

thickness in order to make the scaffold more 3D in nature as well increase the surface area for cell attachment[17].

Hydrogels are a water-swollen, cross-linked network of polymers that are used for cell culture because they mimic extracellular matrices, are mechanically similar to most soft tissues, and can support cell adhesion and growth[18]. There are number of fabrication methods that are used to adjust the internal structure of hydrogels, including free radical polymerization and crosslinking[19]. Hydrogels can be fabricated using synthetic polymers or naturally derived materials. The benefit of using synthetic materials is that the physical properties of the hydrogels can more easily be controlled, such as crosslinking densities and stiffness, while naturally derived materials, including collagen and fibrin, aid in biocompatibility and cell integration of the scaffold[20]. Even though hydrogels are extremely promising when considering cell culture, they do have their own set of fallbacks. Similar to fibrous scaffold, hydrogels can have minimal cell penetration within the scaffold due to the production of small pores during fabrication. Once again this will not allow cells to grow on the full surface area available. A second limitation to hydrogels is that it can be challenging when removing the cells embedded within the scaffold. Detaching cells within the hydrogel can be done by typical mechanical or enzymatic methods, however it takes great care in order to maintain integrity of intracellular components as well as produce sufficient cell yields[18]. It is vital to retrieve as many cells as possible when using hydrogels for cell expansion so this limitation can be a major downfall for the proposed application.

Microcarrier cell culture systems (used interchangeably with microspheres and microbeads) have been used for research purposes since the 1960's for various

applications[21]. Modified microcarriers were used to expand human embryonic cells and to see the effect of cryopreservation on cells adherent to microcarriers, aggregations of microcarrier-based systems were designed to produce microtissues, and research was done on porous microcarriers seeded with stem cells to see the effect on tissue regeneration[22,23,24]. Because the growth of adherent cells is limited to area, the geometry of this scaffold provides a higher surface area-to-volume ratio in comparison to monolayer cell culture, allowing for increased productions and less physical space needed[25]. Microcarriers allow for less cell aggregation and death when compared to cell spheroid formation due to the fact that cells are grown on a monolayer on the scaffold surface when using nonporous options, allowing for more nutrient and waste diffusion[3]. Even though microspheres are advantageous over monolayer and cell spheroid culture, it does require more material and equipment cost during the fabrication process. Also, there are certain fabrication techniques that could prevent the scaffold from being free-floating, which would negate the benefit of there being an increase in area since the entirety of the surface would not be available for cell attachment and growth.

This study focuses on fabricating and testing microcarriers of various diameters to observe if size and curvature have any effect on attachment, metabolic activity, and differentiation of stem cells. There has been successful studies testing different materials and coatings of microcarriers but they tend to focus on one size range of fabricated or commercially bought spheres when conducting cell culture experiments[23,26–28]. Also, research has been conducted using microcarriers as avenues for cell expansion for various cell lines, outlined in Table 1.1. Even though they were able to use coated microcarriers to promote attachment and proliferation, there were still downfalls that need to be

addressed. For the Phillips *et al.* study, cells were able to attach and proliferate when grown on commercially available polystyrene beads, however, the final cell densities were lower than what were previously reported, 5 x 10^5 cells/ml compared to 1.9-3.5 x10^6 cells/mL, which is hypothesized to be caused by a difference in size of microcarriers between the two studies[29]. Heathman *et al.* showed that even though the microcarriers were again successful at promoting a fold- increase in cell quantity, the growth rate decreased during the second half of the cell culture experiment, showing that the agglomeration of the microcarriers could limit the effective surface area available for expansion[30]. This research was established to attempt to optimize these problems in past studies and design an adjustable fabrication system to create multi-sized, homogeneous microcarriers in order to assess the effects of geometry on expanding stem cells for the purposed of advancing stem cell therapies.

Table 1.1 Various microcarrier-based systems for cell expansion

Microcarrier-Based System	Cell Line Tested	Expansion (cells/ml)	Ref.
Trimethylamine-coated Hillex II polystyrene beads (diameter=160-200 μm)	Mouse embryonic stem cells	5 x 10^5 after 5 days (3-fold increase)	29
Fibronectin-coated, non-porous plastic microcarriers (diameter = 160-200 μm)	Human mesenchymal stem cells	3.01x10^5 after 6 days (10-fold increase)	30
Collagen-coated, microporous Cytodex-1 microcarriers under stirred conditions (diameter =147-248 μm)	Sertoli cells	4.5 x 10^5 after 6 days (22.7 fold increase)	31
Poly-e-caprolactone microcarriers coated with vitronectin or laminin (diameter = 148-172 μm)	Human embryonic stem cells	Vitronectin: 1.7 x 10^6 after 7 days (5-7 fold increase) Laminin: 1.1 x 10^6 after 7 days (5-7 fold increase)	32
Cellulose DE-53 cylindrical microcarriers coated with Matrigel under stirred conditions (diameter = 37 μm)	Human induced pluripotent stem cells	6.2 x 10^6 after 7 days (20-fold increase)	33

Microcarriers can be either fabricated in-house or bought for research use. Some commercially available microcarriers are Cytodex (GE Healthcare), Cultispher-G (Percell), and Hillex II (SoloHill), which are proven to be successful in 3D cell culture[28,34,35]. In order to produce microspheres in-house there are a number of methods that can be used including solvent evaporation, phase separation, and spray drying.

Solvent evaporation is one of the most commonly used methods, which involves creating an emulsion between an oil component and a water component. This can either be done as a single emulsion (i.e. oil-in-water, water-in-oil, or oil-in-oil) or a double emulsion (i.e. water-in-oil-in-water). The material, usually a polymer, forms an oil solution by dissolving the polymer particles in an organic solvent such as dichloromethane, acetone, or methylene chloride[36]. This polymer solution then creates an emulsion by mixing it with the water component that contains a surfactant, such as poly (vinyl) alcohol, that stabilizes the emulsion and prevents conglomeration of microcarriers once they are formed in the aqueous solution[36]. This emulsion is then mixed continuously until the solvent has evaporated from the microcarriers and a solid sphere is left[37].

Coacervation, or phase separation, is a process where two liquid phases are used in order to produce microspheres. The first liquid phase is a polymer-rich solution (the coacervate) that is incorporated with a second more dilute polymer solution, which decreases the polymer's solubility[38]. The key to this technique for microsphere fabrication is that the two solutions are oppositely charged and immiscible, so when they are mixed together the separation of phases is induced, creating spheres[39]. A magnetic stirring system is used at appropriate speeds in order to prevent the microcarriers from attaching to one another and forming agglomerations. The formed spheres are hardened

by pouring the mixture into a non-solvent, counter ion solution before washing and drying[36].

The spray drying technique is used for the fabrication of porous and smaller microcarriers with a size range of 1-100 microns depending on the specifications of the nozzle attachment[36]. The polymer chosen for the microcarriers is dissolved in an organic solvent and then used with a laboratory spray-dryer. Spray-dryers include a spray nozzle, an aspirator to provide hot air, a drying chamber, and an area for collecting the dried particles[40]. Within the drying chamber, the polymer solution is sprayed out of the nozzle, producing a fine mist once mixed with the hot gas. Using these commercially available spray dryers, the solvent within the microspheres is immediately evaporated out, and the dried microcarriers are then collected by a filter or cyclone[36].

For this study, polystyrene has been used to fabricate microcarriers for cell expansion experiments using a variation of the solvent evaporation emulsion method. A vibration generator was used in conjunction with a syringe pump in order to create the oil-in-water emulsion between a polystyrene solution and a poly(vinyl) alcohol solution. Changing the frequency of the vibration generator that was used in the fabrication setup was a crucial step when producing different sizes of microcarriers. Traditionally, polystyrene is used in cell culture vessels because it can easily be sterilized by irradiation, has great optical clarity, and is easily moldable into various sizes of flasks, plates, and dishes[41]. In order for cell attachment proteins to adhere and spread more effectively on the polystyrene, the surface is modified to change its properties from hydrophobic to hydrophilic[41]. Briefly, this done by either corona discharge or gas-plasma, which causes

oxygen ions to oxidize and graft onto the surface exposed for treatment, leaving a hydrophilic surface that becomes negatively charged with the addition of media[41].

Because polystyrene flasks go through a treatment process to aid in cell attachment, it was hypothesized that the polystyrene microspheres should also undergo a treatment process to increase adhesion as well. When certain cell lines are grown in adherent 2D cell culture, it is required that the surface is treated with a substrate such as poly-L-ornithine, laminin, fibronectin, or Matrigel to promote attachment, proliferation, and spreading[42]. Without this coating the cells will not attach to the polystyrene cultureware. For this particular study, a previously established peptide solution was used to promote adherent culture on the polystyrene microcarriers. This peptide covalently links to the polystyrene surface and leaves a cell attachment domain available. Most cell lines share a common cell adhesion domain, therefore using this peptide can be applicable for more a multitude of cell types. This is extremely beneficial because the microcarrier-based cell culture system established in this study can be generalize for the expansion of many different cell types.

Neural stem cells (NSCs) were used as an initial model for the microcarrier system developed in this project for cell expansion. Human NSCs are self-renewing and multipotential stem cells, meaning they can divide indefinitely into more cells that are then able to differentiate into all the major cells that make up the adult CNS (neurons, astrocytes, and oligodendrocytes). An ideal treatment for injured brain tissue is to replace the damaged cells with healthy neural cells to restore functionality of the CNS[43]. However, this comes with it own set of disadvantages including an access to a specific amount of cells for an effective transplantation dose, immunological rejection, and lack

of efficacy[44]. Even though there is still room to grow when it comes to improving methods for CNS repair, optimally expanding NSCs to increase the number of viable cells for potential treatments is an important step in the right direction. Using microcarriers for this purpose is beneficial because the increase in surface area due to the geometry of spheres allows for more area for cells to grow and later differentiate into neurons and glial cells.

Table 1.2: Advantages and disadvantages of various systems for 3D cell culture

Method	Description	Advantages	Disadvantages
Forced-floating[45]	Culture wells are coated to prevent attachment, causing the cells to float and later form spheroids by cell-to-cell contact	Simple, reproducible, size is easily adjustable	Extra time/work is required for coatings, increase in cost by buying low-adhesion plates
Hanging drop[45]	Small amounts of cell suspension (~20 μL) are pipetted onto a tray, which is then inverted, forming hanging drops where cells form spheroids at the tip of the drop	Simple, reproducible, low variability in sizes	Cell culture does not allow for volumes larger than 50 μL due to weight of the drop overcoming surface tension. It is also difficult to change media without disturbing the drop
Agitation-based approaches: Spinner flask bioreactors[45]	Spinner flasks form spheroids by continuously mixing the cell suspension with a stirring bar	Simple, large yields of spheroids, easy change of media	Motion causes shear force on the cells which adversely affects physiology, broad range of spheroid sizes
Agitation-based approaches: Rotational culture systems[45]	Spheroids are formed by constantly rotating the culture system about a horizontal axis, preventing the cells from attaching to the system walls	Low shear force, simple, large spheroids yields, easy change of media	Requires specialized equipment, size of spheroids varies
Microfluidic cell culture platforms[45]	Microplates are separated into units including inlet/outlet wells and a culture chamber (CC). The cells are immobilized within the CC, allowing them to form spheroids	Multi-dimensional imaging, minimizing reagent volumes	Retrieval and characterization of spheroids can be difficult
Scaffolds[45]	3D structures, such as hydrogels and microcarriers, offer support for cell growth by use of fibers, pores, and an increase in area	Easily manipulated with cell adhesion molecules, growth factors, or fabrication techniques	Requirement of specialized materials and equipment which can increase cost, retrieval of cells from scaffolds can be difficult

2. MATERIALS AND METHODS

2.1 Materials

Polystyrene (MW~ 350,000), poly(vinyl alcohol) (average MW~30,000-70,000), and goat anti-rabbit IgG antibody were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM) was obtained from Fisher Scientific (Waltham, Massachusetts). Accutase was purchased from Innovative Cell Technologies (San Diego, CA). Alexa Fluor® 488 dye and 4', 6-Diamidino-2-Phenylinole, Dihydrochloride (DAPI) were purchased from Molecular Probes (Eugene, OR). Recombinant human fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) were obtained from Peprotech (Rocky Hill, NJ). Mouse anti-βIII tubulin mAb antibody was purchased from Promega (Madison, WI). Goat anti-mouse IgG1 antibody was purchased from Invitrogen (Rockford, IL). Rabbit anti-GFAP antibody was obtained from Abcam (Cambridge, MA).

2.2 Microcarrier Fabrication

A 15% (wt./vol.) polystyrene (PS) solution was produced by dissolving PS particles into dichloromethane (DCM), an organic solvent not miscible with water. This was done by using a motorized mixer over several hours to allow for a homogenous mixture (Scientific Products, Evanston, IL). A 0.5% (wt./vol. in PBS) poly(vinyl) alcohol (PVA) solution was produced by dissolving PVA in distilled water using a magnetic

stirrer while heated to 105°C and then filtered to remove any debris or particles that were not dissolved.

The fabrication set-up is shown in Figure 2.1A. The PS solution was drawn into a 3 mL syringe (BD, Franklin Lakes, NJ) that was topped with a 23 gauge, 1-inch metal syringe tip (Jensen Global Inc., Santa Barbara, CA). The syringe was securely placed in the syringe pump (World Precision Instruments, Sarasota, FL) and the pump was placed vertically on an adjustable platform so that the syringe tip was pointing directly into a large glass petri dish that was then filled with 200 mL of the 0.5% PVA solution (Figure 2.1A). The syringe was placed underneath the water level to prevent a polymer film forming on top of the PVA solution (Figure 2.1B). The vibration generator (3B Scientific, Hamburg, Germany) was placed behind the glass petri dish so that the vibrating arm was parallel above the dish, intersecting with the syringe tip.

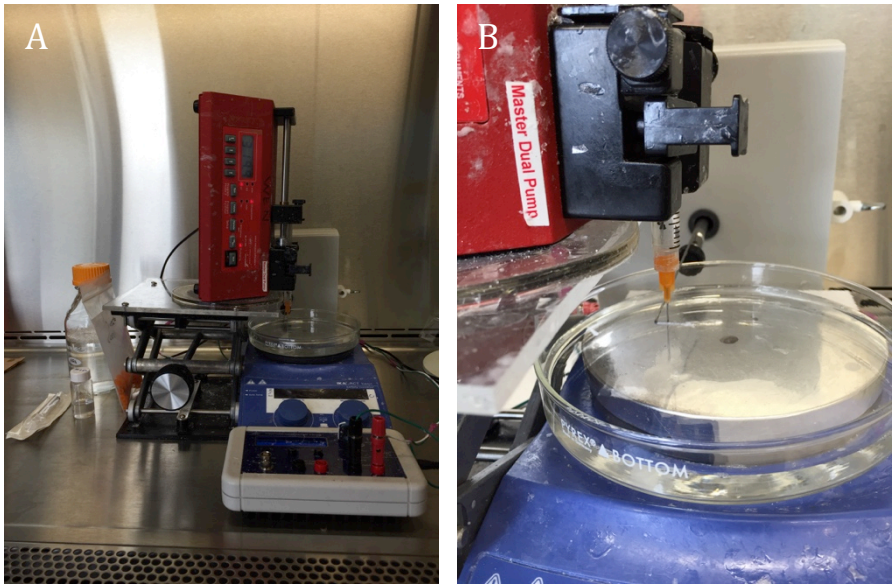

Figure 2.1: A) Complete microcarrier fabrication system including the vibration generator, syringe pump, polystyrene solution within the syringe, and poly(vinyl) alcohol solution within the glass petri dish. B) Close up of the fabrication system.

The syringe pump began pumping at 150 μL/min. Once the air bubbles were released from the syringe, large microspheres began to form and drop into the PVA solution due to an oil-in-water emulsion. A smaller plastic petri dish covered in aluminum foil was placed in the glass petri dish to catch the first several microspheres produced before the vibrations began. Once the larger microspheres were formed regularly, the vibration generator was turned on. The parameters for the vibration generator were as followed: amplitude of 10V, triangle wave, and the frequency varied from 18 Hertz to 24 Hertz. When smaller microspheres were wanted, a higher frequency was used and when larger microspheres were needed a lower frequency was used. After several microspheres were produced, the smaller plastic petri dish was removed and the microspheres began to coat the bottom of the glass petri dish until the 3 mL of PS solution was completely depleted from the syringe, taking about 30 minutes. Each vibration generator was used for 30 minutes followed by a 3-hour rest to prevent overheating. Two vibration generators were used to maximize microsphere fabrication. After the fabrication process, the formed microspheres were allowed to sit in the PVA solution for 2 to 3 days so that the solvent would fully evaporate and the microspheres would harden and become opaque.

2.3 Size Separation

After fabrication and hardening, the microspheres underwent a sieving process to separate them into specific size ranges using different sized test sieves from VWR (Radnor, PA). The glass petri dishes containing the PVA solution and hardened microspheres were poured into a 125-micron sieve and those particles that were smaller

than 125 microns were forced through the sieve by using a plastic squeeze wash bottle filled with distilled water. The microspheres remaining in the 125-micron sieve were then transferred to the 212-micron sieve and the samples smaller than 212 microns were forced through into a beaker using the wash bottle, forming the first size range of 125-212 microns. The aforementioned sieving process was continued until the following size ranges were formed: 125-212 microns, 212-300 microns, 300-355 microns, 355-425 microns, and 425-500 microns. The groups were sieved multiple times to remove any spheres that did not belong in that range. The microspheres were then stored in 50 mL conical tubes in PBS until SEM imaging or cell culture experiments.

2.4 SEM Imaging of Microcarriers

Scanning electron microscope (SEM) imaging was used in order determine the size and 3D topography of the microcarriers. A small amount of microcarriers from each size group were freeze-dried, or lyophilized, in order to prepare the samples for imaging. Briefly, the microspheres were frozen in -80°C and then placed in the Labconco FreeZone® 4.5 Liter Freeze Dry System (Kansas City, MO) overnight. Once fully dried, the microcarriers were securely added to a mount using double-sided tape and then covered with a thin layer of gold making the sample conductive, attracting the electrons within the SEM. The images were obtained using JEOL LV-5610 SEM (Toyko, Japan).

2.5 Human Neural Stem Cell Culture

Human neural stem cells (hNSCs) derived from the ventral midbrain region of the fetal brain were acquired from Millipore (Billerica, MA). These specific hNSCs are a

multipotent human neural progenitor stem cell line that has the ability to differentiate into neurons and glial cells once growth factors are removed from the culture media. hNSCs are self-renewing and immortalized by retroviral transduction with the v-myc oncogene, allowing them to successfully go through numerous passages while maintaining their undifferentiated characteristics. When grown on monolayer plates, it is expected that 100% of the NSCs show expression for nestin before differentiation is induced, which is the marker for neural stem cells[46,47]. When considering undifferentiated NSCs, there should be few or no spontaneous differentiated cells that show expression of beta-III tubulin, the marker for neurons, and GFAP, the marker for astrocytes[46,47]. Seven days after the induction of differentiation, it is normal for there to be a larger percentage of positive staining for beta-III tubulin and a smaller percentage of the cells showing expression for GFAP and O1, the surface marker for oligodendrocytes[46].

An in-house produced peptide mixture (0.04 mg/ml) was used to coat tissue culture flasks for up to 4 hours before cell seeding. The hNSCs were maintained in DMEM/F12 (1:1) medium (Sigma-Aldrich) with added FGF-2 (20 ng/ml) and EGF (20 ng/ml). 2D adherent hNSCs were incubated at 37° C under 5% CO_2 until they reached 80%-90% confluent.

2.6 Microcarrier Cell Culture

After several serial sievings, each size range produced from the microsphere fabrication process (125-212 micron, 212-300 micron, 300-355 micron, 355-425 micron, and 425-500 micron) were separated into two smaller tubes in preparation for surface coatings. The PBS was removed from each tube and the microspheres were washed

multiple times with 75% ethanol to sterilize them prior to cell culture experiments. For the uncoated microcarriers, the samples were washed three times with PBS and then transferred to suspension 48 well culture plates (Sigma-Aldrich, St. Louis, MO) and covered with hNSC media. For the peptide-coated microcarriers, the samples were first coated with 0.01% (wt./vol. in PBS) PVAVS for 10 minutes and then coated with the same peptide solution used in regular 2D culture flasks for up to 4 hours and then placed in the suspension 48 well plates. Suspension plates were used in the experiments to prevent cell attachment to the well surface and promote attachment to the microcarriers. For both the uncoated and peptide coated microcarriers, the same volume of spheres were added to each well to coat the bottom of the surface. In addition to the microcarrier trials, parallel uncoated and peptide-coated 2D samples were run as well. The well plate configuration is shown in Figure 2.2. The hNSCs grown in flasks were rinsed and then incubated with Accutase for 5 min at 37°C to detach the cells from the surface and form a single-cell suspension. After the cell suspension was centrifuged at 220 rcf for 5 minutes, the supernatant was removed and the cells were resuspended in 1 mL of hNSC media to prepare for cell counting using the hemocytometer-based trypan blue dye method. Using this method, it was possible to obtain an accurate count of hNSCs while also obtaining the different percentages of live and dead cells. Both 2D and 3D sample groups were seeded with 40,000 cells/well and the media was changed every other day until Day 7. When necessary, hNSCs grown on microcarriers were dissociated from the surface using the Accustase protocol previously mentioned.

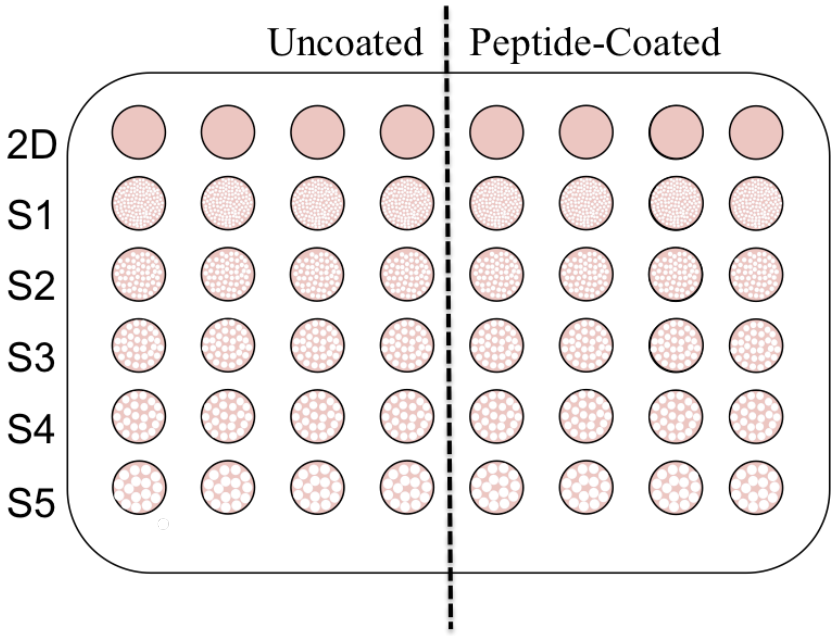

Figure 2.2: Schematic of the microcarrier cell culture experiment.

2.7 Metabolic Activity Analysis

Metabolic activity of the hNSCs were analyzed using the alamarBlue® Assay (Invitrogen). The alamarBlue® Assay is a growth indicator that uses an oxidation-reduction reaction that gives both fluorescent and colorimetric information on the metabolic activity of the samples. In brief, active cells have the ability to continuously reduce nonfluorescent resaurin, the active ingredient in alamarBlue®, into fluorescent resorufin. The more metabolically active cells within the sample, the greater the percent reduction of resazurin and the color of the media will change from blue to bright pink. In this study, the alamarBlue® Assay was used on Day 7 to analyze the metabolic activity of the different samples. Media was removed from all wells, the alamarBlue®/media mixture (1 mL alamarBlue®: 9 mL hNSC media) was added to cover (350 µL), and the well plate was place in the incubator for 4 hours. A control well was included which was

an empty well with no cells filled with the AlamarBlue®/media mixture. After 4 hours, 100 µL of the dye was added in triplicate to a 96 well plate from each study group, including the alamarBlue® control. The 96 well plate was read in the Snergy H1 Multi-Mode Reader (BioTek Instruments, Inc., Winoski, VT) at two wavelengths: 570 nm and 600 nm. The data was then analyzed using the equation provided below to determine percent reduced in each study group.

$$\% \text{ Reduced} = \frac{(\varepsilon_{ox})\lambda_2 A\lambda_1 - (\varepsilon_{ox})\lambda_1 A\lambda_2}{(\varepsilon_{red})\lambda_1 A'\lambda_2 - (\varepsilon_{red})\lambda_2 A'\lambda_1} \text{ x } 100$$

With the following parameters:

$\varepsilon_{ox} =$ molar extinction coefficient of alamarBlue® oxidized form,

$\varepsilon_{red} =$ molar extinction coefficient of alamarBlue® reduced form,

$A =$ absorbance of test wells, $A' =$ absorbance of negative control wells, $\lambda_1 =$ 570 nm, $\lambda_2 = 600$ nm, $\varepsilon_{ox}\lambda_1 = 80{,}586$, $\varepsilon_{ox}\lambda_2 = 117{,}216$, $\varepsilon_{red}\lambda_1 = 155{,}677$, and $\varepsilon_{red}\lambda_2 = 14{,}652$.

2.8 Immunofluorescence

After the AlamarBlue® Assay analysis, the cells were washed with PBS, fixed with 4% (wt./vol. in PBS) paraformaldehyde (PFA) and then permeabilized with 0.2% (vol./vol. in PBS) Triton-X 100 for an additional 30 minutes. For cell attachment studies, F-actin was stained with Alexa Fluor® 488 dye (5 µL dye: 200 µL PBS) for 2 hours. After 2 hours, the nuclei were then counterstained with DAPI (1 µL dye: 1000 µL PBS) for 10 minutes. For differentiation studies, the cells were fixed and permeabilized as mentioned previously. The cells were then blocked with 10% (vol./vol.) goat serum in PBS for 30 minutes. The samples were sequentially incubated with the primary

antibodies for neurons and glial cells overnight at 4° C and then incubated with the secondary antibodies for 1 hour at room temperature. The nuclei were counterstained with DAPI for 10 minutes. For neuronal staining, the primary antibody used was mouse anti-βIII tubulin mAb (G7121) and the secondary antibody used was goat anti-mouse IgG1 (A-21121, 1:400). For glial staining, the primary antibody used was rabbit anti-GFAP (ab7260) and the secondary antibody goat anti-rabbit IgG TRITC (T6778, 1:400). To characterize the neural stem cells, nestin colocalization was confirmed by using the primary antibody Mouse Anti-Nestin Antibody (Millipore, MAB5326, 1:100) and the secondary antibody goat anti-mouse Cy2-conjugated Affinipure, IgG/FCγ1 (Jackson ImmunoResearch, 15100). The groups were finally washed three times with PBS and then covered in a 0.1% sodium azide solution in order to preserve the samples. Microcarriers were imaged over various time periods using an Olympus CKX41 culture microscope (Melville, NY) to observe cell attachment. Fluorescent images of the stained undifferentiated and differentiated hNSCs were acquired with an Olympus IX81 confocal microscope.

2.9 Statistical Analysis

Statistical analysis was performed in MATLAB using one-way analysis of variance (ANOVA) followed by Tukey's post-tests. A probability (p) value of < 0.05 was considered statistically significant. The data is shown as mean $\pm$ standard deviation.

3. RESULTS

3.1 Microcarrier Fabrication

Polystyrene microspheres were fabricated using a variation of the oil-in-water emulsion technique through a programmable vibration generator in combination with a syringe pump. The overall goal of the fabrication process was to optimize the size distribution and homogeneity of the scaffold. The factors controlling the size of the microcarriers included concentration of the polystyrene solution, thickness of the syringe tip, rate of the syringe pump, and vibration generator parameters. The most successful factor when producing various sizes of microcarriers was the parameters of the vibration generator within the fabrication system, mainly the frequency of the vibrating arm. When the frequency of the arm was lower (17-19 Hz), the microcarriers formed were larger and more uniform (Figure 3.1A) and when the frequency was higher (19+Hz) the spheres were less homogeneous and smaller (Figure 3.1B).

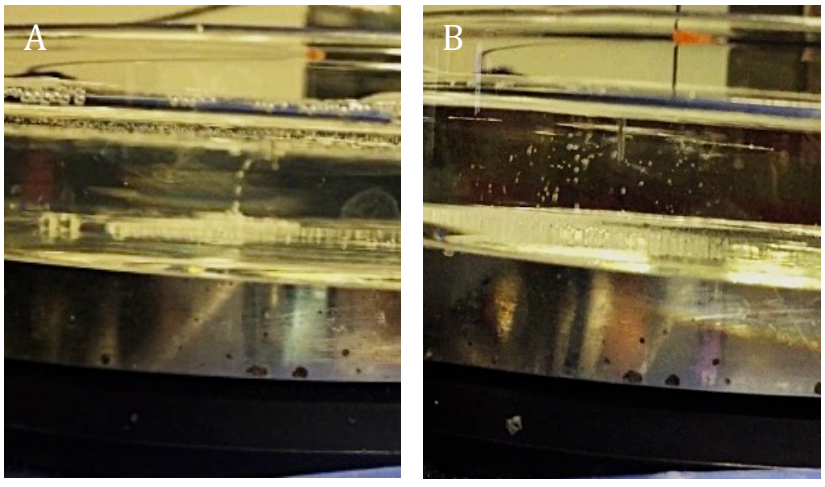

Figure 3.1: The diameter of the polystyrene microcarriers decreased and became less homogeneous when the frequency of the vibrating arm increased. A) Microcarriers when the vibrating arm was set to 18 Hz. B) Microcarriers produced when the vibrating arm was set to 22 Hz.

3.2 Characterization of Polystyrene Microcarriers

After fabrication and hardening, the microspheres underwent a multi-step sieving process in order to separate the samples into specific size spectrums. From this process, five different sizes ranges were produced which are shown in Table 3.1. A small amount of microspheres were taken out of the stock batches and freeze-dried for SEM imaging in order to give a more accurate determination of the size and topography (Figure 3.2). For each size range, multiple SEM images were taken and 100 microspheres were analyzed. The images were opened in ImageJ, the scale was set using the legend bar given on the figure, and the area of the spheres were measured using the circle tool. This area measurement was then converted to diameter dimensions in Excel, giving the average diameter data shown in Table 3.1.

Table 3.1: Size characterization of fabricated microcarriers

	Size Range *(um)*	Average Diameter *(um)*
Size 1 (S1)	125-212	165.41$\pm$ 21.80
Size 2 (S2)	212-300	256.27 $\pm$ 20.46
Size 3 (S3)	300-355	317.33 $\pm$ 26.65
Size 4 (S4)	355-425	407.52 $\pm$ 26.36
Size 5 (S5)	425-500	470.39 $\pm$ 35.42

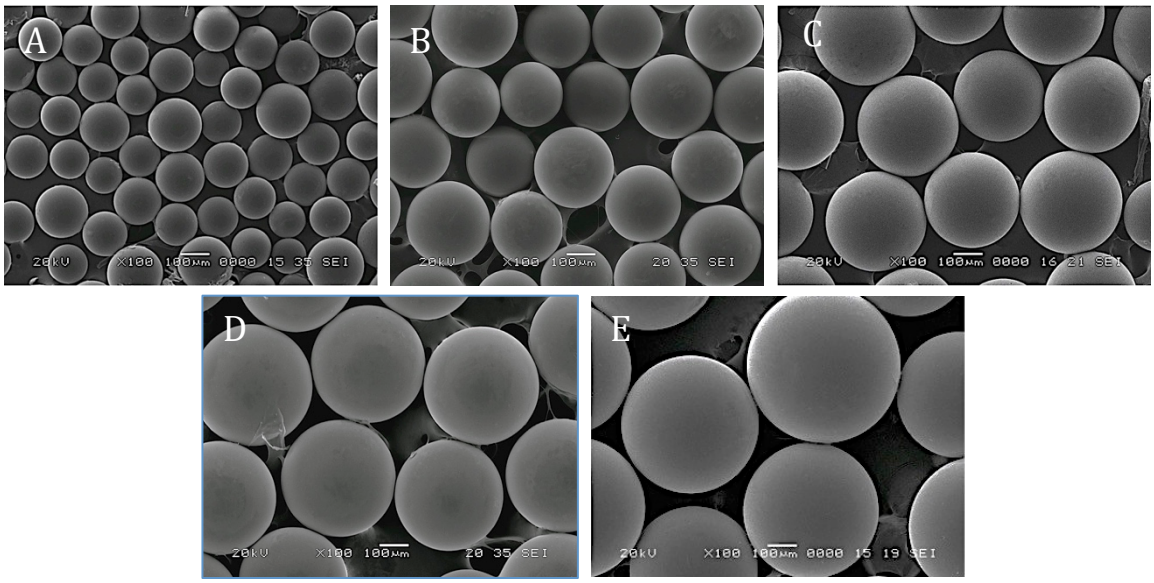

Figure 3.2: Microcarriers were produced using a vibration generator in conjunction with a syringe pump in order to create an oil-in-water emulsion between the polystyrene solution and the poly(vinyl) alcohol solution and then separated using various sized sieves. (A) Size 1 (125-212 microns), (B) Size 2 (212-300 microns), (C) Size 3 (300-355 microns), (D) Size 4 (355-425 microns), (E) Size 5 (425-500 microns).

In the cell culture experiments, roughly the same volume of microcarriers for each size spectrum was used for each well (~0.12 cm^3), assuming random sphere packing. The total surface area and volume of one microcarrier was calculated with the formulas $V_s = \frac{4}{3}\pi r^3$ and $SA_s = 4\pi r^2$. The total amount of spheres per well for each sample size was then determined by dividing the total volume per well by the volume of one sphere. This was used to determine the surface area-to-volume ratio for each size spectrum, shown in Figure 3.3. Size 1 (125-212 microns) had the highest ratio while Size 5 (425-500 microns) had the lowest ratio, which was expected because the smaller the diameter the larger the SA-to-V ratio.

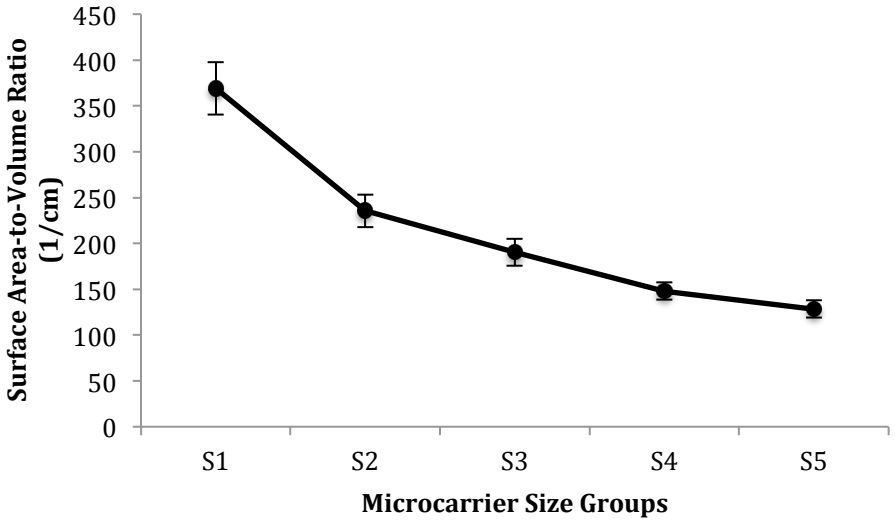

Figure 3.3: As the diameter of the microcarrier increased, the surface area-to-volume ratio decreased. Size 1 (d=165.41± 21.80 microns) has the highest SA-to-V ratio (369.09 cm^{-1}) while Size 5 (d=470.39 ± 35.42 microns) has the lowest SA-to-V ratio (128.25 cm^{-1}).

3.3 Stem Cell Attachment to Microcarriers

Both optical and confocal imaging were used in order to visualize the ability of hNSCs to attach and remained attached to the peptide-coated microcarriers while also not adhering to the surface of the well plate. Immediately after cell seeding, the cells are seen floating in the culture media (Figure 3.4 A, D, G, J, M) and by Day 2 the cells have attached to the surface of the microcarriers and formed junctions between the spheres (Figure 3.4 B, E, H, K, N). The culture experiment was continued for 7 days before the cells were fixed and then stained with DAPI (nucleus) and phalloidin (actin), further showing stem cell attachment (Figure 3.4 C, F, I, L, O). By Day 7, the cells within the wells of the uncoated microcarriers had been washed away by changing of media and there was minimal attachment to the surface of the spheres (Figure 3.5).

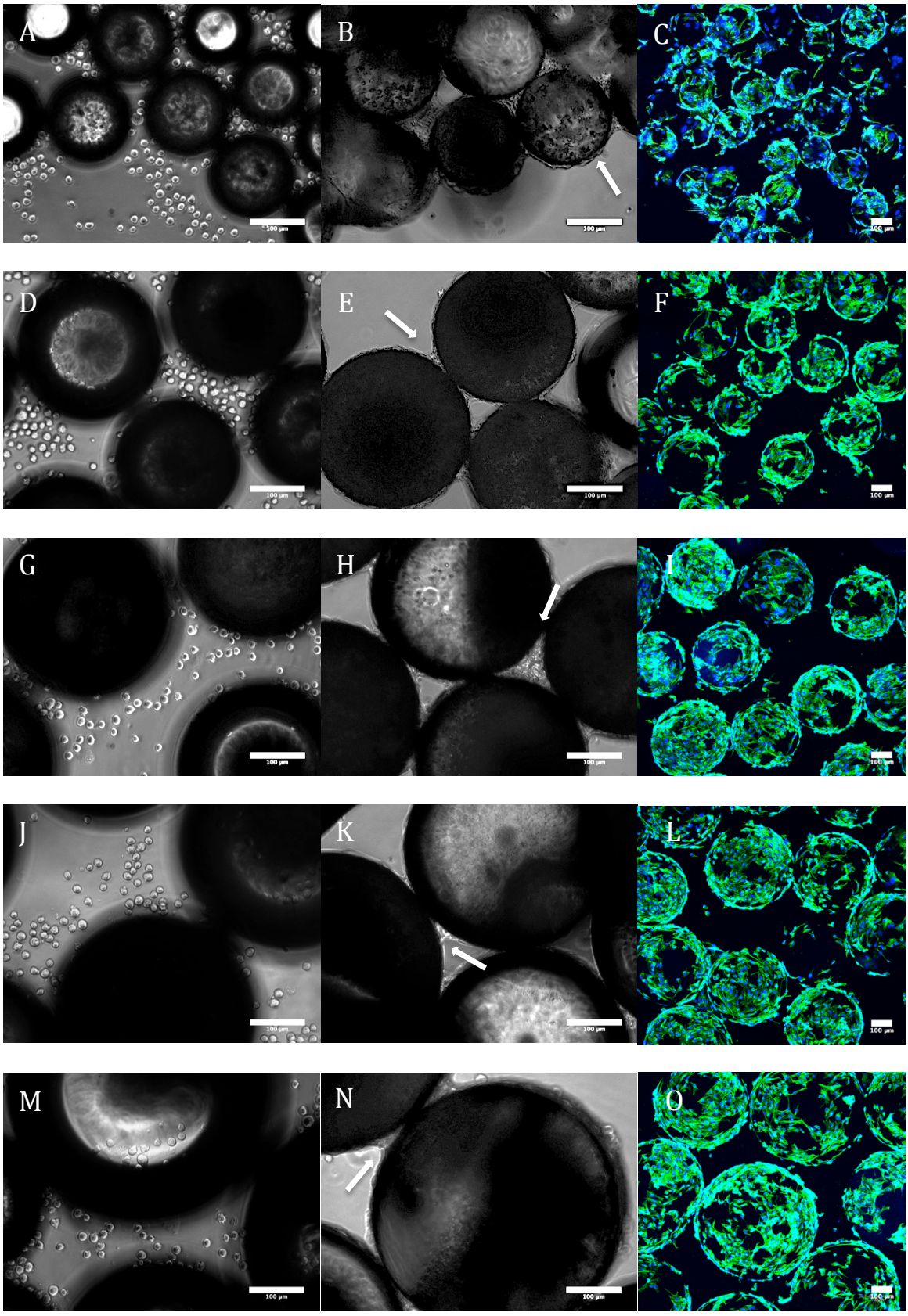
A
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Figure 3.4: Peptide-coated microcarriers were able to promote human neural stem cell attachment to the surface of the spheres while in suspension well plates, which prevented cell attachment to the well surface. Size 1 (A-C), Size 2 (D-F), Size 3 (G-I), Size 4 (J-L), Size 5 (M-O). Optical images immediately after cell seeding (A, D, G, J, M) and 48 hours after seeding (B, E, H, K, N). Merged confocal images 7 days after the start of the experiment, with the nucleus stained with DAPI (blue) and actin stained with phalloidin (green) (C, F, I, L, O). All scale bars: 200 microns.

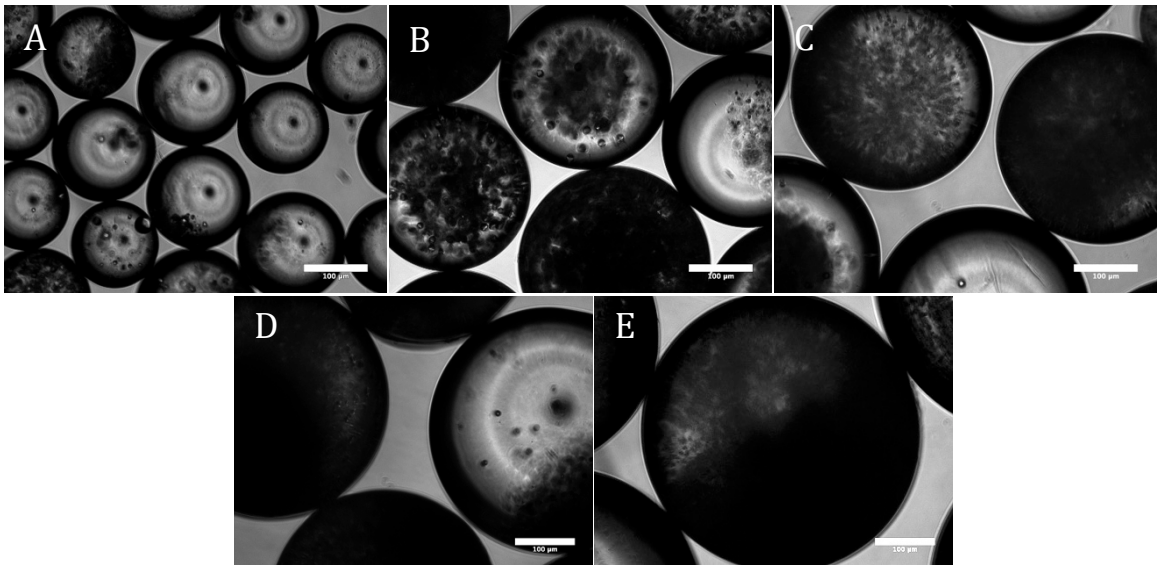

Figure 3.5: Uncoated microspheres at Day 7. There was no significant stem cell attachment to Size 1 (A), Size 2 (B), Size 3 (C), Size 4 (D), or Size 5 (E). Scale bar: 200 microns.

After confocal imaging, the DAPI images (Figure 3.6 A-F) of the peptide-coated 2D and 3D samples, which respresent the hNSC nuclei, were analyzed in ImageJ to determine fluorscent intensity followed by cell count. Cell count was normalized by surface area available with that image, shown in Figure 3.6G. When seeding with the same density of hNSCs, the 2D and 3D samples resulted in similar cells per mm^2, further showing that microcarriers are able to support cell attachment and growth..

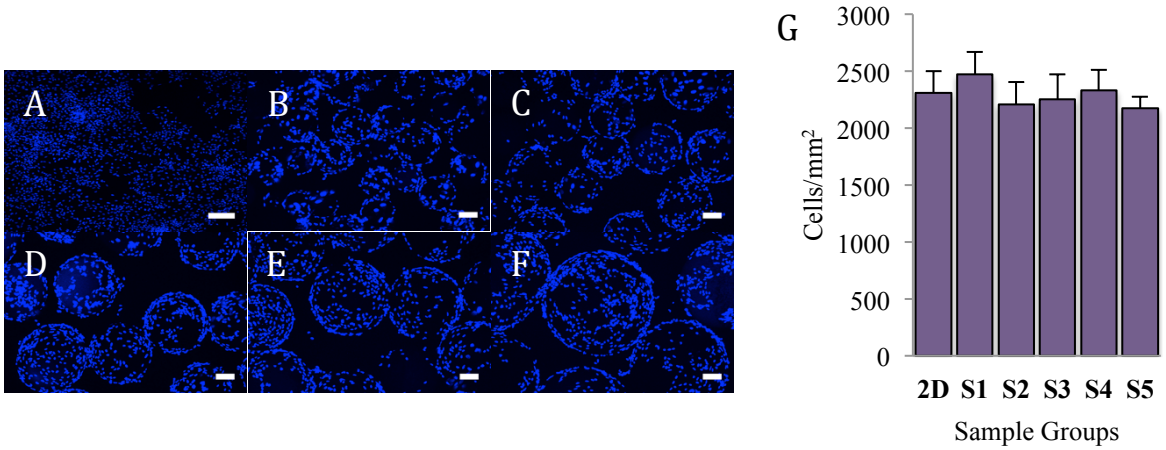

Figure 3.6: The nuclei of neural stem cells grown on a peptide-coated monolayer (A) as well as on the varying diameters of peptide-coated microcarriers (B-F) were stained with DAPI and then analyzed in ImageJ to determine the number of cells per area. The 2D and 3D samples were statistically similar when comparing cells/mm^2, further showing that the microcarriers aided in the attachment and spreading of cells when there was a substrate coating. Scale bar: 100 microns.

3.4 Metabolic Activity Analysis

After 7 days in cell culture, the alamarBlue® Assay was performed in order to assess the cell viability of hNSCs grown in monolayer culture and on the polystyrene microcarriers. The higher the percent reduction of alamarBlue®, the more metabolically active cells within that well. For the five size ranges as well as the 2D control, there was both an uncoated sample group as well as a peptide-coated group. For each pair of samples, the uncoated group was statistically lower than the peptide-coated group (Figure 3.7), giving evidence that a beneficial coating is necessary to promote cell adhesion for adherent neural stem cell culture. When comparing all the peptide-coated groups, there were similarities between the 2D and 3D samples (Figure 3.8), giving further evidence that the increase in area of the microcarriers promotes cell growth without hindrance from the surface curvature. However the 2D sample was statistically higher than S3-S5,

which could be due to loss of microcarriers and therefore loss of hNSCs during media renewal.

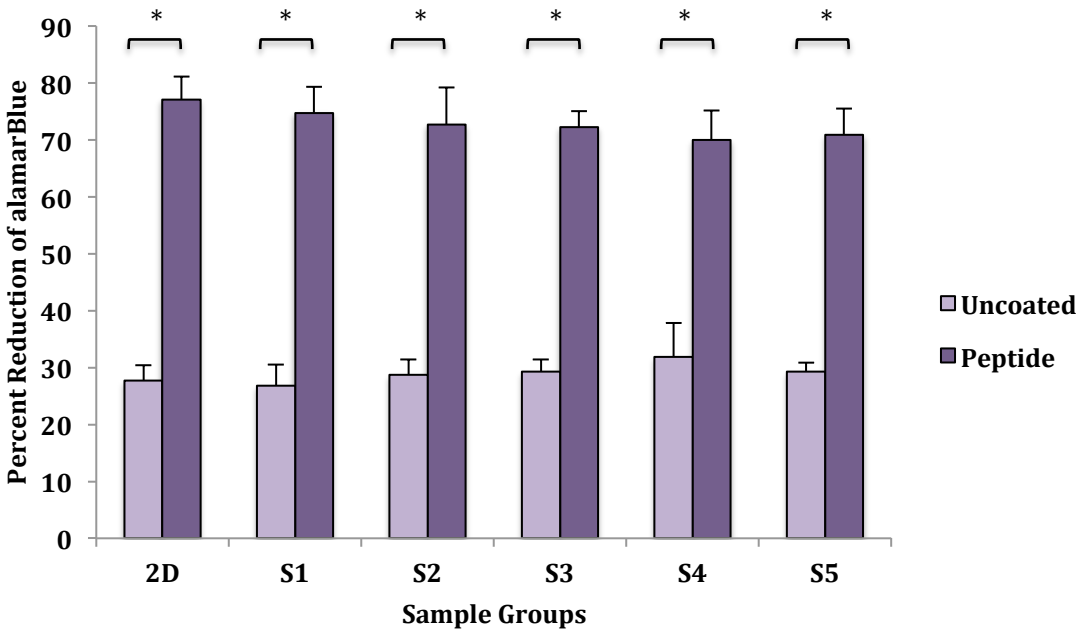

Figure 3.7: The alamarBlue® Assay was used to assess metabolic activity of the microcarrier cell culture experiments. The more metabolically active cells within the sample, the greater the percent reduction of resazurin, the active ingredient within alamarBlue®. For each microcarrier size as well as the 2D control, the uncoated sample component was statistically less when it came to percent reduction compared to its peptide-coated counterpart, showing the benefit of the peptide coating on cell adhesion.

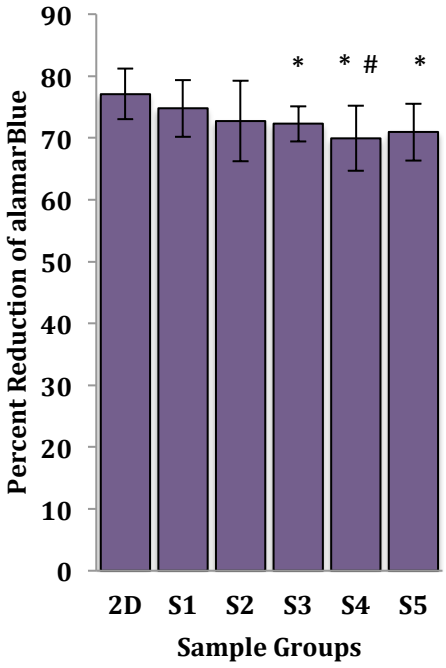

Figure 3.8: When comparing the alamarBlue® Assay results for the 2D and 3D peptide-coated samples, a majority of the groups were statistically similar, showing that the coated microcarriers were able to promote cell attachment and growth while also allowing the cells to remain viable and metabolically active. * represents a significant difference compared to the 2D group and # represents a significant difference compared to the S1 group.

3.5 Differentiation of Human Neural Stem Cells

Neural stem cells attached to peptide-coated microcarriers were grown in hNSC media without the addition of growth factors in order to induce neuron and glial cell differentiation. For this differentiation study, S1 (125-212 microns), S3 (300-355 microns), and S5 (425-500 microns) were used. After 12 days, the multipotency of hNSCs grown in a three dimensional manner was confirmed by using antibody staining against specific proteins within neurons and glial cells (Figure 3.9). During this time, cells not only grew around the curvature of the microcarriers, but also formed junctions

of cell bodies in between the spheres (Figure 3.9 E, I, M), which was not possible during monolayer culture (Figure 3.9 A-D).

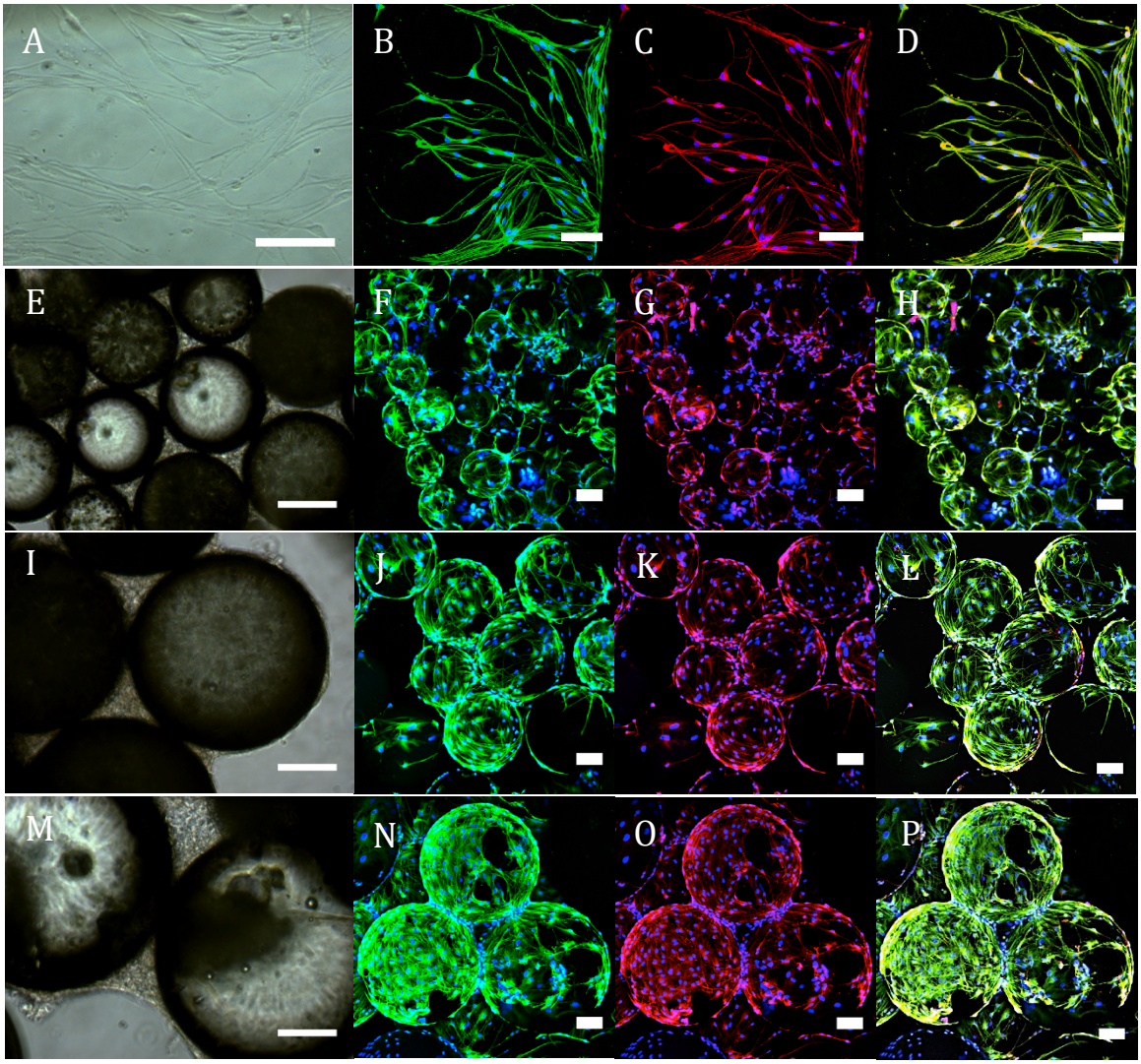

Figure 3.9: Peptide-coated microcarriers were able to provide support for hNSC differentiation to neurons and glial cells after 12 days in differentiation media. 2D monolayer (A-D), Size 1 (125-212 micron) (E-H). Size 3 (300-355 microns) (I-L). Size 5 (425-500 microns) (M-P). While in differentiation cell culture, neural cells began forming cellular masses in between microcarriers (E, I, M). Cell nuclei were stained with DAPI (blue), differentiated neurons were labeled with anti-Beta III tubulin antibodies (green, column 2), and differentiated glial cells were labeled with anti-GFAP antibodies (red, column 3). Column 4 represents the merge of all three dyes. All scale bars: 100 microns.

4. DISCUSSION

Pharmacological treatments of neurodegenerative disorders and neural injuries are available to relieve some symptoms for patients, however, a move intensive method is needed for long-term repair of the central nervous system[48]. In order to make positive strides towards successful therapies for degenerative diseases there needs to be a practical method to provide a large number of neural stem cells in a timely manner. Microcarriers are currently being used as a scaffold for adherent cell culture to aid in this expansion procedure because they provide a larger surface area per unit volume compared to monolayer culture. When considering cell expansion, a large surface area-to-volume ratio is advantageous because not only is there more area for cells to attach and proliferate, the microcarriers also take up less space[25]. Longer culture times, higher cell density and effective scalability are also beneficial when using three-dimensional scaffolds in comparison to normal flask culture.

For this experimental study, multi-sized polystyrene microcarriers were produced using a vibration generator and solvent evaporation techniques in order to grow and expand human neural stem cells (hNSCs) in a three dimensional manner. The main objective of this project was to produce homogenous spheres of varying diameters to promote cell attachment, proliferation, and differentiation. Different combinations of

syringe gauge tips, syringe pump rates, and frequency of the vibration generator were used to determine how the size of the microcarriers would respond. The thicker syringe tips typically produced larger microcarriers while thinner syringe tips had a tendency to produce smaller spheres. Also the higher the frequencies (20 Hz and higher), the smaller and less homogenous the microspheres while larger and more consistent spheres were produced at lower frequencies (18 Hz and below). When it comes to particle size, the successful fabrication of microcarriers with varying diameters can be beneficial for different applications. Smaller spheres sizes have been used for smaller stirred flasks while larger microcarriers can be used in industrial-sized bioreactors for cell culture given their higher sedimentation rate due to a higher mass[25]. To further expand this study, the microcarrier fabrication system and materials can be modified so that the microcarriers are free floating within the media without the use of agitation, which can cause damaging shear force upon the cells. This will allow the entirety of the microsphere's area to be available for cell attachment compared to when beads are coating the bottom of the culture plates. This can be adjusted by changing the combination of materials used during the fabrication process, such as using a base solution that is miscible with dichloromethane like acetic acid.

Polystyrene was used in this study because of its optical clarity, biocompatibility, ease of molding into various structures, and customary use in a laboratory setting. In order to enhance cell adhesion to the polystyrene, the samples were coated with a peptide solution. This is advantageous in the long run because the cell attachment domain within the peptide sequence can be used to adhere various cell lines, making this process open for more than just NSC culture. Future studies will use different cell lines, such as human

induced pluripotent stem cells or human mesenchymal stem cells, to compare the expansion rates. The addition of a peptide coating on both the 2D monolayer samples as well as all five microsphere sizes proved successful in aiding cell attachment of neural stem cells 24 hours after cell seeding without the addition of agitation. The cells remained attached to the microcarriers for up to 7 days before fixing and staining. In order to amplify the study, staining against nestin can be used in future studies in order to fully characterize the neural stem cells that have attached the peptide-coated microcarriers. Typically when NSCs are grown in undifferentiated media in two dimensions, 100% of the cells stain positive for the nestin marker so it would be beneficial to compare this quantity to the percentage of cells expressing nestin when grown on peptide-coated microcarriers[46,47]. In addition, to verify the viability of the cells grown on the microcarriers, the LIVE/DEAD Viability Kit should be used, which uses green fluorescent SYTO 10 to stain live cells and red fluorescent ethidium homodimer-2 to label dead cells. The more viable cells retrieved, the more applicable the microcarriers will be for stem cell therapies.

Any uncoated samples showed minimal attachment of hNSCs through optical imaging, further proving the need for a favorable cell adhesion coating for the polystyrene microcarriers. Cells grown on the peptide-coated microcarriers maintained their metabolic activity and were comparable to peptide-coated 2D wells. In addition, any uncoated sample possessed a statistically lower amount of proliferation compared to their parallel peptide-coated groups. This is a positive outcome because it shows that when microcarriers are modified with an advantageous coating they are able to promote cell growth without the curvature of the spheres causing a negative effect. However, when

comparing certain peptide-coated microcarrier sizes to the 2D samples, there was some discrepancy between the percent reductions of alamarBlue®, showing that the monolayer culture had more metabolically active cells compared to the 3D culture when seeding with the same amount of cells. Even though the numbers were similar, the difference could be due to loss of microcarriers in cell culture during media renewal or agglomeration of microcarriers during cell growth. During future studies, this process will be optimized to prevent microcarrier and cell loss in order to get the most accurate metabolic activity data.

In order for hNSCs to be used for future clinical studies involving CNS therapies, they need to maintain their differentiation abilities while undergoing this expansive process. In this study, hNSCs were able to differentiate into neurons and glial cells without any hindrance from surface coating or surface curvature when grown on the fabricated polystyrene microcarriers of varying diameters. Neurons are responsible for processing and transmitting information within the CNS while glial cells are responsible for supporting the functions of neurons, so the greater the number of hNSCs that are expanded through this process and then eventually differentiated in neurons and glial cells, the more useful this research will be to the end goal of successful neural cell transplantation[49]. To further analyze this differentiation experiment, future studies will include fluorescent-activated cell sorting, or FACS, to determine the percentage of cells that express neuron and astrocyte markers within the population of differentiated cells grown on the microcarriers. Typically for NSC culture, a larger portion of the differentiated cells show expression for beta-III tubulin, the marker for neurons and a smaller percentage of cells showed expression for GFAP, the marker for astrocytes[46]. So

to prove that microcarriers systems not only support but also have a positive effect on differentiation, is would expected that the percentage of expression for neurons and astrocytes would be similar or greater than what is typically seen in NSC cell culture. Also, a control group will be included where undifferentiated NSCs grown on microcarriers are stained against beta-III tubulin and GFAP to determine the baseline of neurons and astrocyte expression. In typical NSC culture, undifferentiated cells showed close to zero expression for beta-III tubulin and GFAP[46,47].

During the differentiation experiments, the cells attached to the surface and spread around the curvature of the spheres as well as formed cellular masses through cell-to-cell interactions, causing the microcarriers to began to agglomerate. This suggests that microcarriers allow cells to grow in a three dimensional manner as cellular bodies while also growing as a monolayer on the surface of the spheres, which is an advantage to traditional flat surfaces because it creates a more *in-vivo* like environment. However, the cell masses that begin to form in between microcarriers should be carefully watched because if the cell density becomes too high the transport of nutrients and waste will become inefficient and the hNSC may loss their natural abilities to proliferate and later differentiate.

In order to continue to optimize this system, the polystyrene microcarriers should be used in larger vessels with varying seeding densities to see how long the scaffold can support stem cell growth. This study showed that 2D and 3D samples were consistent when it came to the amount of attachment and metabolically active cells once the 2D samples became confluent, however, it is expected for the 3D samples to have a higher cell quantity in the long-term due to the limited surface area of monolayer culture and the

increase of area in the microcarrier samples. When considering 100% confluency, an average yield is around 10^5 cells/cm^2. For a 48 well plate, which was used in this study, each 2D well is expected to yield about 80,000 cells. Moving forward, the 2D and 3D samples should be monitored after seeding with 80,000 cells to see the effect of the increase in area of the microcarriers. In an ideal setting, S1 (average surface area in this study (SA_{avg}) = 34.8 cm^2) will produce a cell yield of 3.5 x 10^6, S2 (SA_{avg}= 22.6 cm^2) will produce a cell quantity of 2.3 x 10^6, S3 (SA_{avg}= 19.0 cm^2) will produce a cell quantity of 1.9 x 10^6, S4 (SA_{avg}= 13.6 cm^2) will produce a cell quantity of 1.4 x 10^6, and S5 (SA_{avg}= 11.8 cm^2) will produce a cell quantity of 1.2 x 10^5, shown in Figure 3.10. Therefore, long-term studies are hypothesized to show the benefit of microcarrier-based systems over monolayer systems through an increase in cell quantities as well as show the benefit of using smaller diameter microcarriers due to the increase in surface area available for cell attachment and growth.

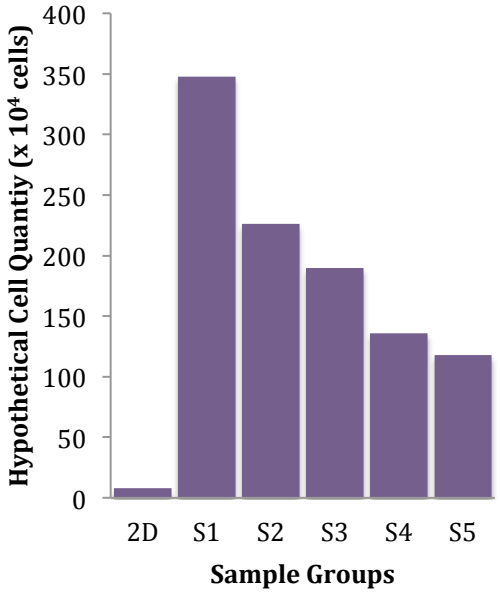

Figure 3.10: Hypothetical cell quantities for 2D and 3D samples once 100% confluency have been reached.

5. CONCLUSION

In this study, homogenous polystyrene microcarriers were successfully produced using an oil-in-water emulsion and vibration generator in order to promote hNSC attachment, viability, and differentiation. The fabrication technique was optimized to produce microcarriers of varying diameters. These microcarriers provided a three-dimensional environment for cell growth as well as an increase in surface area, both advantageous when compared to the traditional flat tissue culture plates and dishes. When supplemented with a beneficial peptide surface coating, cell attachment and proliferation was significantly amplified without any hindrance due to surface curvature. After 12 days of being cultured in differentiation media, the hNSCs attached to the microcarriers were effectively differentiated into neurons and glial cells, which is the ultimate goal for central nervous system injury treatments by cell transplantation. This research demonstrates the growing potential of microcarrier-based cell culture systems on future stem cell therapies through rapid cell expansion.

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